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TARGETED THERAPY FOR VIRAL HEPATITIS

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Summary

New compounds for the therapy of chronic viral hepatitis, such as nucleoside analogs, antisense oligonucleotides or ribozymes, have been developed in the past several years. Although many are effective inhibitors of viral replication *in vitro*, efficacy *in vivo* and further clinical applications is often hampered by broad biodistribution and extraheptic toxicity after systemic administration. Because the liver is the primary site of infection and damage in viral hepatitis, specific targeting of antiviral drugs to hepatocytes may be useful. For this purpose, particle-type carriers such as liposomes and endogenous lipid particles, as well as soluble drug carriers, especially ligands of the highly liver-selective asialoglycoprotein receptor, are currently under investigation. The use of these compounds could result in the efficient delivery of various new agents to the liver, and in the clinical application of new antiviral drugs in the future.

Introduction

Infections with hepatitis B virus (HBV), hepatitis C virus (HCV) and hepatitis D virus (HDV) cause a substantial burden on health care resources worldwide. Current estimates suggest that there are 200-300 million HBV carriers worldwide (1, 2), with the highest prevalence in Southeast Asia and sub-Saharan Africa, where up to 20% of the population is infected (3, 4). HDV infection only occurs in association with HBV. However, its prevalence is much lower and does not parallel HBV infection, probably accounting for less than 5% of chronic viral hepatitis (1, 5). It is found in approximately 5% of HBsAg carriers (6). HCV is the main cause of post-transfusion hepatitis. Its worldwide prevalence varies and is reflected in the prevalence of HCV antibodies in the blood donor population, ranging between 0.3% (in the United Kingdom and Scandinavia) and 3.6% (in Eastern Europe and parts of Africa). Because the prevalence in the general popu-

lation is thought to be higher, current estimates suggest that approximately 200 million people worldwide (7, 8) and 3.5 million or 1.4% of the population in th U.S. (1) have chronic HCV infection.

The clinical picture in patients with chronic infection varies from the healthy carrier state to acute or chronic liver disease leading to liver cirrhosis and liver failure. Chronic viral hepatitis due to HBV or HCV infection and liver cirrhosis are the main risk factors for development of hepatocellular carcinoma (9-11). The morbidity and mortality of chronic viral hepatitis have necessitated a prolonged search for effective therapy. Despite rapid advances in the understanding of the molecular biology of the viruses, only α-interferon is approved for therapy of HBV and HCV infection. Although useful in selected patients, the overall rate of sustained remission is only 30-40% for HBV (12), 15-30% for HCV (6, 13) and even lower for HDV (14), making the development of new approaches a major concern.

Conventional antiviral drugs such as nucleoside analogs or new compounds such as antisense oligonucleotides or ribozymes, which are effective inhibitors of viral replication *in vitro*, are often hampered *in vivo* because of broad biodistribution or extrahepatic toxicity. Drug targeting offers different advantages over conventional administration of drugs. High local concentrations of the drug can be achieved and distribution to tissues where its presence is not required can be avoided, thus circumventing many unwanted sid effects. Specific delivery of antiviral drugs to the cell type where viral replication takes place is a challenging approach. Even though peripheral blood mononuclear cells have been suggested as extrahepatic reservoirs for HBV and HCV, recent results show little evidence for active replication (15, 16). Because the liver is the primary site of infection and damage in viral hepatitis, specific targeting of antiviral drugs to hepatocytes may be useful.

Basically two different approaches have been employed to achieve selective delivery of antiviral compounds (17, 18): 1) the use of prodrugs, which modulate the biodistribution characteristics and pharmacokinetic properties of the original agent to improve the availability of a drug at a desired cell type, and 2) carriers, both particulate and soluble, with the same rational. This review will focus on the use of carriers as tools for drug targeting to the liver.

Particle-Type Carriers as a Targeting Device

The category of particle-type drug carriers includes liposomes, naturally occurring lipid particles, microspheres and nanoparticles. Yet, the specific d livery of antiviral agents to hepatocytes has been accomplished by the use of liposomes and lipid particles like LDL, HDL and chylomicrons.

Liposomes

Liposom s are microscopic vesicles consisting of one or multiple phospholipid bilayers surrounding one or multiple aqueous compartments. Liposome clearance from the circulation is a function of their size and surface composition. It must be noticed that the pores within th fenestrated wall of endothelial cells in the liver have a diameter of about 100 nm (19). Therefore, particles exceeding this size, as well as molecules larger than 250 kD can not pass into the space of Disse and, therefore, do not interact significantly with hepatocytes (18, 20). For this reason, liposomes larger than 100 nm are cleared by phagocytosis through Kupffer and endothelial cells. An advantage of liposomes as targeting device is the fact that drugs can be simply incorporated, either dissolved in the aqueous phase or associated with the lipid material, without covalent linkage (18). In addition, the encapsulated compound is protected against enzymatic degradation.

One of the first reports about drug delivery to hepatocytes by liposomes showed an enhanced antiviral effect of murine interferon entrapped in liposomes compared to the free compound against murine hepatitis virus in mice (21). The fate of intravenously injected liposomes depends largely on their lipid composition (22). By using small unilamellar liposomes consisting of cholesterol/egg phosphatidylcholine (1:1), 2/3 of the total dose injected intravenously in rats accumulated in the liver and 97% of this amount was found in parenchymal cells (23). To reduce the toxicity and to deliver a higher proportion of the administered drug to the liver, Hostetler and coworkers incorporated different antiviral nucleosides in liposomes consisting of phosphatidylcholine, phosphatidylglycerol and cholesterol. Liposomal phosphatidyl-dideoxycytidine (ddC) and liposomal phosphatidyl-dideoxythiacytidine (3TC, lamivudine) showed less cytotoxicity and only slightly reduced antiviral activity *in vitro* (24, 25) compared to the free drug, but had a 40-fold higher drug level in the liver after intraperitoneal administration in mice (24). Most recently, the same group showed that liposomal phosphatidyl-dideoxyguanosine (ddG), intraperitoneally injected in woodchucks, inhibited serum levels of woodchuck hepatitis virus (WHV) DNA 23- to 46-fold after 4 weeks of daily administration compared to 2.2- to 10.4-fold after treatment with free ddG (26).

To enhance the capacity of targeting liposomes to parenchymal liver cells, various ligands recognized by receptors on the liver cell surface were incorporated in the liposomal phospholipid bilayer. Examples for such targeting moieties are epidermal growth factor (27), lactosylceramide (28, 29), asialofetuin (30, 31), lactose mono-fatty acid esters (32,

33) and β-galactoside (34). By mploying asialofetuin-coated liposomes to encapsulate interferon-γ, an increase of the inhibitory effect on the replication of HBV-DNA in Hep-HB107 cells compared to noncoated liposomes has been shown (31).

For many preparations, uptake by endothelial or Kupffer cells compared to parenchymal cells is still predominant, and there is no unanimity on the quantitative aspect of the differential uptake into different cell types in the liver (22). Liposomes with galactose residues are also recognized by Kupffer cells via the galactose-particle receptor (18), and the distribution between parenchymal and nonparenchymal liver cells is strongly size-dependent (35), with only very small liposomes with limited loading capacity or vesicles containing lactosylceramide (28) or lactose mono-fatty acid esters (32, 33) preferentially directed to parenchymal cells. Furthermore, while nucleosides, antisense-oligonucleotides or ribozymes act in the cytoplasm of the target cells, these modified liposomes enter cells through an endocytotic mechanism, followed by transfer to the degradative lysosomal compartment. Compounds that cannot escape this pathway may not be therapeutically active (36, 37). Presently, little is known about the intracellular fate of agents delivered to liver cells by targeted liposomes. Even though some encouraging results for the use of liposomes in the treatment of chronic viral hepatitis are emerging, further difficulties might arise with industrial large-scale production (38).

Endogenous lipid particles

Endogenous lipid particles, like LDL, HDL and chylomicrons, consist of lipid and apoprotein components. By glycosylation of the protein part or incorporation of glycolipids in the lipid part, cell-specific recognition by receptors other than the physiological r ceptors can be achieved. The incorporation of a triantennary galactose-terminated cholesterol derivative in HDL particles directed the conjugate to parenchymal liver cells (39). In a related work it was shown that the degree of lactosylation of LDL particles is important for the distribution of uptake between Kupffer and parenchymal cells, with a higher degree of lactosylation resulting in a preferential uptake by Kupffer cells (40). After lactosylation of the protein moiety of natural (41) or synthetic neo-HDL particles (42) and intravenous injection into rats, 95% and 80%, respectively, accumulated in the liver and uptake by parenchymal cells accounted for 98% and 90%, respectively, of the total liver uptake (41, 42). Since the preinjection of an excess amount of *N*-acetylgalactosamine or asialofetuin blocked the uptake in the liver, it was concluded that internalization is mediated by endocytosis via the asialoglycoprotein receptor. More recently, recombinant chylomicrons were successfully used to deliver the nucleoside analog iododeoxyuridine via the largely liver-specific apolipoprot in E r ceptor (43) to parenchymal liver cells (44). Nevertheless, there are no reports about the antiviral activity of nucleoside analogs targeted to liver cells by the use of naturally occurring lipid particles or neolipids.

Soluble Glycoprotein Carriers as a Targeting Device

The major difference of soluble carriers compared to the former type of carrier is the smaller size of soluble carriers, with molecular weights below 200 kD. Therefore, they can easily pass through the fenestrated endothelial lining of liver capillaries and interact directly with hepatocytes. The asialoglycoprotein receptor (45), present in large numbers only on hepatocytes, binds galactose-terminated glycoproteins and neoglycoproteins with high affinity. Bound ligands are internalized by the cell via receptor-mediated endocytosis. Due to its specificity, the asialoglycoprotein receptor (AsGPr) has been exploited as a means to deliver drugs (46-48) and DNA (49-51) for therapeutic purposes, as well as diagnostic agents (52-54) to hepatocytes.

Targeting of nucleoside analogs

In 1979, Fiume *et al.* (55) demonstrated a specific delivery of trifluorothymidine (F3T), conjugated to asialofetuin, to hepatocytes. Conjugation was achieved by coupling the *N*-hydroxysuccinimidyl ester of F3T glutarate with the ε-amino groups of asialofetuin. It was shown to interact specifically with the AsGPr and to inhibit DNA synthesis in *Ectromelia* virus-infected liver cells selectively.

Subsequent studies by the same group have focused on adenine arabinofuranoside monophosphate (ara-AMP) and acyclovir monophosphate conjugates with galactosylated albumin (56-59). Albumin was galactosylated with lactose by reductive amination. The conjugates were synthesized with a phosphoramide bond between the drug and ε-amino groups of the protein. The conjugates interacted specifically with the AsGPr and released the drug within liver cells (60). When prepared with homologous albumin, the conjugates were not immunogenic (61). Later the coupling reaction was modified to obtain predominantly monomeric conjugates to optimize specific delivery to parenchymal liver cells (62, 63). In woodchucks with viral hepatitis, conjugated ara-AMP or conjugated acyclovir inhibited viral replication at a dose 8- to 13-fold lower than the free drug (64). The appearance of intracellular vacuoles in mouse and rat liver cells after adminis-

tration of the conjugate was shown to result from lysosomal swelling (65). The effect disappeared aft r cessation of treatment and was prevented by lowering the dose. In the first studies in patients with chronic HBV infection, administration of the conjugate for 3-7 days showed a marked, but not sustain d decrease of plasma HBV-DNA levels comparable to that of free drug, but at a dose 3-6 times lower (66, 67). No side effects were observed. In the latest study of the same group (68), 8 patients were treated with a higher dose over a period of 4 weeks. One patient developed small amounts of antibodies against the conjugate, but no other significant side effects were observed. All patients responded with a decrease of plasma HBV-DNA levels, but levels rose again after discontinuation of treatment.

The need for intravenous infusion of galactosylated araAMP prompted the search for other syst ms for targeting nucleoside analogs to hepatocytes. Conjugates with lactosylated poly-L-lysine also have been shown to target the AsGPr, and they were used to deliver ara-AMP (69), ribavirin and azidothymidine (70) to the liver after intramuscular administration in mice, and 5-iodo 2'-deoxyuridine after intravenous injection in rats (71). In woodchucks with viral hepatitis, ara-AMP conjugated to galactosylated poly-L-lysine showed comparable effects after intramuscular administration compared to ara-AMP conjugated with galactosylated albumin after intravenous administration (72). More recently, similar results were reported for a conjugate of ara-AMP with arabinogalactan, a naturally occurring polysaccharide and ligand of the AsGPr, when used to treat woodchuck hepatitis virus-infected woodchucks (73, 74).

Targeting of antisense oligonucleotides

In the last few years, antisense oligonucleotides and ribozymes have been explored as a new class of pharmaceuticals to inhibit the replication of hepatitis viruses. A number of antisense sequences which are capable of inhibiting the replication of hepatitis B (75-77) and hepatitis C (78, 79) viruses *in vitro* have been identified. Efficacy has also been observed with an antisense phosphorothioate DNA *in vivo* (80). Very recently, ribozymes have been shown to be effective against hepatitis B (81-83) and hepatitis C (84) gene expression. However, since cellular uptake of oligonucleotides is generally poor, and their susceptibility to degradation in plasma can be quite high, some form of targeting would be desirable for successful use of antisense and ribozyme strategies for therapy of viral hepatitis *in vivo*.

A system based on asialoglycoprotein-poly-L-lysine conjugates has been developed to target DNA or various other compounds to the liver via the AsGPr (46-50). α_1 Acid glycoprotein, orosomucoid, was desialylated by tr atm nt with neuraminidase to produce asialoorosomucoid (ASOR), a high-affinity ligand for the AsGPr. Poly-L-lysine (PL) was then covalently attached to the protein by carbodiimide-mediated amide bond formation. The resulting ASOR-PL conjugate bound the negatively charged DNA in a nondamaging electrostatic interaction (85) and protected it from nuclease degradation (86). The complex was selectively and rapidly internalized into hepatocytes by receptor-mediated endocytosis, and foreign genes were expressed *in vitro* (49) and *in vivo* (50).

The same strategy was used to prepare ASOR-PL complexes with an 21-mer antisense oligonucleotide complementary to the sequence of the polyadenylation signal of the HBV genome. By using a radioactive end-labeled species, it was determined that the oligo alone was taken up with a rate of 0.05 pmol/h/million cells by two hepatoma cell lines, HepG2 (AsGPr-positive) or SK Hep 1 (AsGPr-negative). However, the uptake of oligo conjugated to ASOR-PL was 10 times faster into HepG2 cells, but was not changed in SK Hep 1 cells. Coincubation with an excess asialoorosomucoid blocked the uptake. To show whether the targeted antisense has antiviral activity, the HepG2 2.2.15 cell line was used. This cell line possesses AsGPrs, is stably transfected with the complete HBV genome and secretes viral antigens as well as infectious virus particles. Administration of complexed antisense DNA blocked the expression of HBsAg in these cells and reduced the replication of viral DNA by about 80% compared to untreated controls. A complexed oligonucleotide with random sequence had no effect, and the antisense oligo DNA alone decreased the expression of surface antigen and viral replication by only approximately 30% (87).

In a subsequent investigation, ASOR-PL complexed to a 21-mer phosphorothioate antisense oligonucleotide against the polyadenylation region and adjacent upstream sequences of WHV was used to treat WHV-infected woodchucks. Animals were injected intravenously with ASOR-PL complexes containing 0.4 mg antisense for 5 consecutive days (total dose 2 mg/animal, 0.1 mg/kg/day). Although there was no difference in the levels of surface antigen between treated and untreated animals, a significant decrease in viral burden was observed. Treated animals showed a 1-2 log decrease in circulating viral DNA 25 days posttreatment. The decline lasted for approximately 2 weeks, after which there was a gradual rise in DNA levels. Antisense alone or a complex containing a random oligo DNA of the

same size and linkage failed to have any significant effect on viral DNA levels (88).

Targeted pretreatment of hepatocytes with the above antis nse oligonucleotide complexed to ASOR-PL was used to prevent subsequent infection with HBV. Usually, it cannot be anticipated wh n an acute exposure to HBV will occur. Howev r, after liver transplantation in patients infected with HBV, the grafts are invariably reinfected. Furthermore, there is an accelerated course in most cases. Protection of the graft by pretreatment could prevent reinfection. Pretreatment of Huh7 cells (AsGPr-positive) with ASOR-PL antisense complexes before lipofection with an HBV-plasmid (6.5 million copies of plasmid per cell) inhibited the amount of newly synthesized, core-associated viral DNA in Huh7 cells to undetectable levels, or less than 0.1 pg, as assessed by quantitative PCR. HBsAg, secreted by the cells into the medium, was inhibited in a dose-dependent manner by a maximum of 97%, and the inhibition lasted for 6 days. Pretreatment with unconjugated antisense or complexed random oligo showed no significant effects (89).

Very recently, a related targeting device, consisting of human adenovirus particles conjugated to *N*-acetyl-glucosamine-modified bovine serum albumin, streptavidin and PL, was used to deliver phosphorothioate-modified 16-mer antisense oligonucleotides to hepatocytes via the AsGPr. The oligonucleotide was directed against the encapsidation signal of the core gene. Chicken hepatoma cells (LHM) were transfected by complexed HBV-DNA. When the cells were treated with complexed oligonucleotide before and after treatment with complexed HBV-DNA, an approximately 80% inhibition of core-particle-associated HBV-DNA levels was observed (90).

Conclusions

In the last few years, many new approaches have been developed to treat chronic viral hepatitis. These include novel nucleoside analogs, inducers of viral specific nucleases, interferon, thymic peptides, ribozymes and antisense oligonucleotides. However, toxic side effects in nontarget tissues are often dose-limiting, and a broad biodistribution reduces efficient concentrations in the liver. Targeting through the use of receptor-mediated endocytosis of these new agents could circumvent these drawbacks in the future.

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